Myosin light chain kinase binding to actin filaments

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Abstract Smooth muscle myosin light chain kinase (MLCK) plays important roles in contractile-motile processes of a variety of cells. Three DFRxxL motifs at the kinase N-terminus (residues 2–63) are critical for high-affinity binding to actin-containing filaments [Smith et al. (1999) J. Biol. Chem. 274, 29433–29438]. A GST fusion protein containing residues 1–75 of MLCK (GST75-MLCK) bound maximally to both smooth muscle myofilaments and F-actin at 0.28 and 0.31 mol GST75-MLCK/mol actin with respective K_D values of 0.1 μM and 0.8 μM. High-affinity binding of MLCK to actin-containing filaments may be due to each DFRxxL motif binding to one actin monomer in filaments.

Key words: Myosin light chain kinase; Myofilament; Actin; Calmodulin; Calcium

1. Introduction

Smooth muscle contraction is regulated by myosin light chain kinase (MLCK), a Ca^{2+}/calmodulin-dependent enzyme [2]. Contraction is initiated by an increase in intracellular Ca^{2+}, which binds to calmodulin (CaM). Ca^{2+}/CaM forms a ternary complex with MLCK, facilitating activation of the kinase and phosphorylation of the 20 kDa regulatory light chain of myosin. Phosphorylation induces a conformational change, which allows myosin crossbridges to progress along actin filaments. Some non-muscle motile processes such as platelet aggregation and contraction [3–5], fibroblast contraction [6,7] and mast cell secretion [8] are also regulated by myosin light chain phosphorylation.

The N-terminus of the kinase binds to actin filaments in vitro and in vivo [1,9,11–15]. A core sequence containing three DFRxxL repeat sequences located in residues 1–75 was both necessary and sufficient for binding to smooth muscle myofilaments [1]. Because of their structural identity, we considered the possibility that each motif may bind one actin monomer in an actin filament. Additionally, it is not clear if these motifs are sufficient for binding to purified F-actin alone compared to detergent-washed smooth muscle myofilaments or stress fibers in cells in culture [1]. Therefore, quantitative studies were performed on the binding properties of a glutathione *S*-transferase (GST) fusion protein containing residues 1–75 of the smooth muscle MLCK (GST75-MLCK) to purified F-actin and smooth muscle myofilaments.

2. Materials and methods

2.1. Construction of phosphorylatable GST75-MLCK

Polymerase chain reaction was used to engineer a GST75-MLCK with a C-terminal phosphorylation site corresponding to the recognition site for protein kinase A, using the following primers: 5′-GAGAATGGTAGCTGCGACGAGCGTCGCTGGGACCCGGGTCG-3′ and 5′-GGCGCACCTAGGTACCTAAAG-3′. The primers were designed as the non-coding strand of rabbit smooth muscle MLCK. PCR reactions were performed for 35 cycles of denaturing, annealing and extension (95°C, 45 s; 63°C, 30 s; and 72°C, 30 s). Reactions were then incubated at 72°C for 10 min. The final PCR product was digested with *Bam*HI and *Sma*I and subcloned into pGEX-4T-2 (Amersham Pharmacia Biotech).

2.2. Protein expression

Recombinant plasmid containing GST75-MLCK was transformed into BL21(DE3) cells. The cells were incubated with shaking at 37°C until a density of 0.6 was obtained at A_{595}. Protein induction was achieved by incubating cells an additional 3 h in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested at 6000×*g* for 15 min, resuspended in 5 ml phosphate-buffered saline, 5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 10 μg/ml pepstatin, and lysed by sonication at 4°C. The lysed suspension was then centrifuged at 2000×*g* for 10 min at 4°C, the supernatant fraction was filtered with a 0.45 μm filter (Nalgene), and GST fusion protein was purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) affinity column chromatography. Purified protein was dialyzed against 50 mM MOPS, pH 7.0, 1 mM $MgCl_2$, 1 mM dithiothreitol and 10% glycerol, and stored at −80°C.

2.3. Phosphorylation of GST75-MLCK

GST75-MLCK (25–50 μM) was phosphorylated at the C-terminal sequence LRRASLG by the catalytic subunit of protein kinase A (5 μg/100 μl reaction) in the presence of 100 μM ATP (15 μCi) in 10 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, and 1 mM dithiothreitol. Most of the unincorporated ATP was removed with spin columns containing buffer used in the binding assays (Bio-Rad).

2.4. Extraction of smooth muscle myofilaments

Chicken gizzards (2 g skinned and ground) were homogenized in 10 ml of 10 mM MOPS, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol using a polytron homogenizer (Brinkmann Instruments). Homogenized tissue was centrifuged at 17 320×*g* for 10 min at 4°C. The pellets were homogenized in 10 ml of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 50 mM $MgCl_2$, and 3% Triton X-100 to release endogenous MLCK. The homogenized suspensions were centrifuged at 17 320×*g* for 10 min, at 4°C. After washing in the detergent-containing buffer five times, the myofilaments were then washed three times in 10 mM MOPS, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol and resuspended in 6 ml of the same buffer and stored on ice.

2.5. Myofilament binding

To assay MLCK binding to smooth muscle myofilaments, 0.70 mg/ml of washed myofilaments was mixed with phosphorylated GST75-MLCK (0–3 μM) in a final volume of 150 μl. The binding buffer

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Abbreviations: MLCK, myosin light chain kinase; CaM, calmodulin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GST, glutathione *S*-transferase

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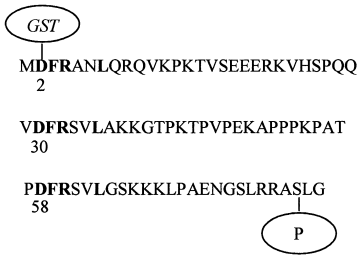

Fig. 1. GST75-MLCK construct. The three DFRxxL motifs in the 75 N-terminal residues of smooth muscle MLCK are highlighted in bold. The sequence encoding a GST fusion protein was added to the N-terminus of residues 1–75 of smooth muscle MLCK while a protein kinase A phosphorylation site (LRRASGL) was inserted at the C-terminus.

contained 10 mM imidazole, pH 7.2, 50 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA, 10% glycerol and 0.2 mg/ml bovine serum albumin. The mixtures were incubated on ice for 30 min and centrifuged at $17\,320\times g$ for 5 min at 4°C. An aliquot of the supernatant fraction was spotted on cellulose filter paper, washed in phosphoric acid to remove residual free ATP. A similar washing treatment was performed with the total reaction mixture, and the radioactivities were then measured by liquid scintillation spectrometry. The decrease in radioactivity in the supernatant fractions compared to radioactivity in the total reaction mixture was used to calculate bound GST75-MLCK. Incubation of GST75-MLCK with myofilaments did not result in dephosphorylation by protein phosphatase activity (data not shown). Actin content in myofilament preparations was estimated from Coomassie-stained gels after SDS–PAGE.

2.6. Actin binding

F-actin from rabbit skeletal muscle was prepared [16]. Various concentrations of phosphorylated GST75-MLCK (as described for myofilament binding) were mixed with 3 μM purified F-actin, and incubated at 25°C for 20 min in the buffer described for myofilament binding. The mixture was centrifuged at $150\,000\times g$ for 1 h and 4°C. ^{32}P-labeled GST75-MLCK remaining in the supernatant fraction was determined as described above.

3. Results

3.1. MLCK structure

All known vertebrate smooth muscle MLCKs contain three DFRxxL motifs identified as necessary for actin binding by smooth muscle MLCK in vitro and in vivo within the first 75 residues of the kinase [1]. Therefore, we used a GST fusion protein containing residues 1–75 of MLCK with a phosphorylation site at the C-terminus of the peptide to measure binding affinity and stoichiometry quantitatively after labeling with ^{32}P (Fig. 1). This method is more sensitive than Western blotting after SDS–PAGE, allowing a large range of concentrations.

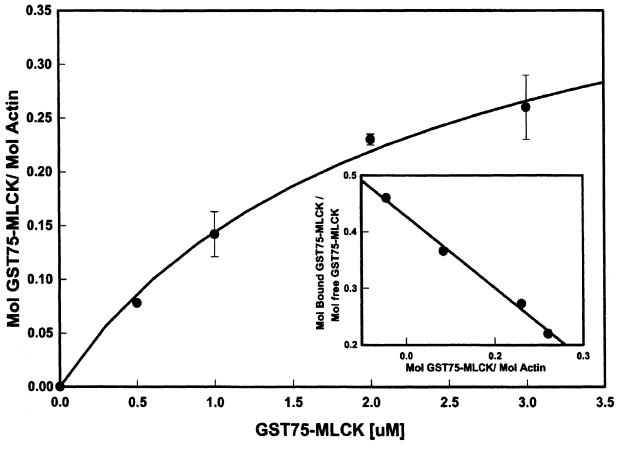

Fig. 2. GST75-MLCK binding to purified F-actin. Phosphorylated GST75-MLCK (0–3 μM) was mixed with 3 μM actin as described in Section 2. The GST75-MLCK bound (mol/mol actin) is plotted against GST75-MLCK concentration. A Scatchard plot of the data is shown in the inset.

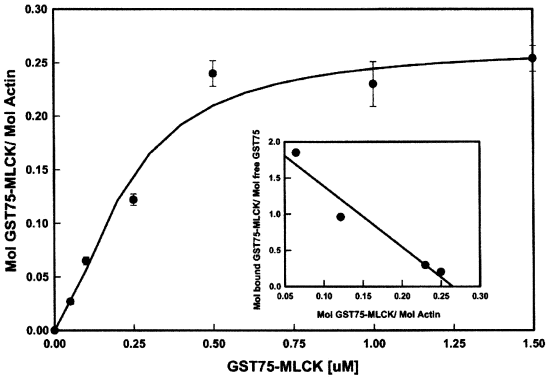

Fig. 3. GST75-MLCK binding to smooth muscle myofilaments. Phosphorylated GST75-MLCK (0–3 μM) was mixed with 3 μM actin in myofilaments as described in Section 2. The amount of GST75-MLCK bound (mol/mol actin) is plotted against GST75-MLCK concentration. A Scatchard plot of the data is shown in the inset. The conditions for the binding assay are as described in Fig. 2 except 0.70 mg/ml myofilaments were substituted for F-actin.

3.2. F-actin binding

To measure GST75-MLCK binding to F-actin, various concentrations of ^{32}P-labeled fusion protein were incubated with 3 μM F-actin. Maximal binding was obtained at 0.31 mol GST75-MLCK/mol actin (Fig. 2, Table 1). The apparent K_D value was 0.80 μM (Fig. 2, Table 1), which is 3–20-fold less than that reported by others [11,12,14,15,17,18]. It is possible that variability in actin affinities may be due to binding assay conditions. We compared several buffers frequently used in actin cosedimentation assays and the buffer used herein resulted in consistently lower K_D values (data not shown).

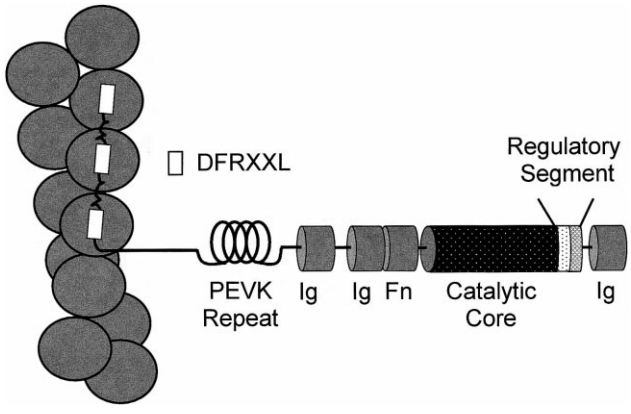

Fig. 4. Schematic model for GST75-MLCK binding to actin-containing filaments. There are sufficient intervening residues between each DFRxxL motif to span from one actin monomer to another actin monomer in the filament. The stoichiometries of binding to both purified F-actin and actin in smooth muscle myofilaments support a model for the association of each DFRxxL with one actin monomer (see text for details).

Table 1
Properties of GST75-MLCK binding to actin-containing filaments

Peptide	Binding substrate	B_{max} (mol GST75-MLCK/mol actin)	K_D (μM)
GST75-MLCK	F-actin	0.31 ± 0.04	0.80 ± 0.11
GST75-MLCK	Myofilaments	0.28 ± 0.02	0.12 ± 0.024

3.3. Binding of GST75-MLCK to smooth muscle myofilaments

Comparable to F-actin binding, the maximal amount of GST75-MLCK bound to myofilaments was 0.28 mol GST75-MLCK/mol actin with an apparent K_D of 0.12 μM (Fig. 3, Table 1). This K_D value is lower than the K_D value for binding to purified F-actin.

4. Discussion

An N-terminal peptide of smooth muscle MLCK is sufficient for high-affinity binding to actin-containing filaments. This construct contains the three DFRxxL motifs as well as flanking residues, but does not include the downstream repeat sequence, Ig-like or Fn-like motifs. The DFRxxL sequences encompass a part of the amino-terminal sequence of 75 residues which is highly conserved in smooth muscle MLCK, but this sequence is not found in skeletal muscle MLCK which does not bind actin-containing filaments [10,12]. The observation that GST75-MLCK displays a greater affinity for smooth muscle myofilaments than purified F-actin suggests that other associated proteins such as tropomyosin or caldesmon may increase affinity. It is not clear if GST75-MLCK is binding only to actin in the intact myofilaments or if the binding is facilitated by these or other accessory proteins.

Based on the binding stoichiometry with both purified F-actin and smooth muscle filaments, we propose a model for association of the three DFRxxL sequences of MLCK with actin-containing filaments (Fig. 4). Circular dichroism spectrometry shows no secondary structure in an expressed MLCK peptide containing residues 1–108 plus a His tag which binds to actin filaments (Zhi and Stull, unpublished observations). Assuming no secondary structure, the calculated maximal linear distance between the aspartate residues in each of the DFRxxL motifs is about 120 Å. The radius of each actin monomer is about 90–95 Å [18–20]. The defined spacing probably positions the kinase such that each DFRxxL motif binds to one actin monomer with the combined binding energies resulting in high-affinity binding. Importantly, the immobilized kinase bound to actin filaments phosphorylates myosin regulatory light chain in thick filaments [12]. The DFRxxL sequences may be important in localizing the kinase to various compartments within the cell, according to localization of F-actin with and without accessory proteins. However, the relative portion and functional consequences of bound versus unbound kinase in cells is not yet established.

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References

[1] Smith, L., Su, X.J., Lin, P.J., Gang, Z. and Stull, J.T. (1999) J. Biol. Chem. 274, 29433–29438.
[2] Stull, J.T., Lin, P.J., Krueger, J.K., Trewhella, J. and Zhi, G. (1998) Acta Physiol. Scand. 164, 471–483.
[3] Mamiya, S., Hagiwara, M., Inoue, S. and Hidaka, H. (1989) J. Biol. Chem. 264, 8575–8579.
[4] Hashimoto, Y., Sasaki, H., Togo, M., Tsukamoto, K., Horie, Y., Fukata, H., Watanabe, T. and Kurokasa, K. (1994) Biochim. Biophys. Acta 1223, 163–169.
[5] Stull, J.T., Krueger, J.K., Kamm, K.E., Gao, Z.-H., Zhi, G. and Padre, R. (1996) in: Biochemistry of Smooth Muscle Contraction (Barany, M., Ed.), pp. 119–130, Academic Press, Orlando FL.
[6] Holzapfel, G., Wehland, J. and Weber, K. (1983) Exp. Cell Res. 148, 117–126.
[7] Kolodney, M.S. and Elson, E.L. (1993) J. Biol. Chem. 268, 23850–23855.
[8] Choi, O.H., Adelstein, R.S. and Beaven, M.A. (1994) J. Biol. Chem. 269, 536–541.
[9] Guerriero Jr., V., Rowley, D.R. and Means, A.R. (1981) Cell 27, 449–458.
[10] Nunnally, H.M. and Stull, J.T. (1984) J. Biol. Chem. 259, 1776–1780.
[11] Sellers, J.R. and Pato, M.D. (1984) J. Biol. Chem. 259, 7740–7746.
[12] Lin, P.J., Luby-Phelps, K. and Stull, J.T. (1997) J. Biol. Chem. 272, 7412–7420.
[13] Lin, P.-J., Luby-Phelps, K. and Stull, J.T. (1999) J. Biol. Chem. 274, 5987–5994.
[14] Ye, L.H., Hayakawa, K., Kishi, H., Imamura, M., Nakamura, A., Okagaki, T., Takagi, T., Iwata, A., Tanaka, T. and Kohama, K. (1997) J. Biol. Chem. 272, 32182–32189.
[15] Kudryashov, D.S., Chibalina, M.V., Birukov, K.G., Lukas, T.J., Sellers, J.R., Van Eldik, L.J., Watterson, D.M. and Shirinsky, V.P. (1999) FEBS Lett. 463, 67–71.
[16] Pollard, T.D., Goldbert, I. and Schwarz, W.H. (1992) J. Biol. Chem. 267, 20339–20345.
[17] Kanoh, S., Ito, M., Niwa, E., Kawano, Y. and Hartshorne, D.J. (1993) Biochemistry 32, 8902–8907.
[18] Hayakawa, K., Okagaki, T., Ye, L.H., Samizo, K., Higashi-Fujime, S., Takagi, T. and Kohama, K. (1999) Biochim. Biophys. Acta 1450, 12–24.
[19] Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Nature 347, 37–44.
[20] Holmes, K.C., Popp, D., Gebhard, W. and Kabsch, W. (1990) Nature 347, 44–49.